Hyperhomocysteinemia but Not the C677T Mutation of Methylenetetrahydrofolate Reductase Is an Independent Risk Determinant of Carotid Wall Thickening

The Perth Carotid Ultrasound Disease Assessment Study (CUDAS)

Brendan M. McQuillan, MBBS; John P. Beilby, PhD, FAACB; Mark Nidorf, MD, FRACP; Peter L. Thompson, MD, FRACP; Joseph Hung, MB, FRACP

Background—Hyperhomocysteinemia has been identified as a potential risk factor for atherosclerosis. This study examined whether a modest elevation of plasma total homocysteine (tHcy) was an independent risk factor for increased carotid artery intimal-medial wall thickness (IMT) and focal plaque formation in a large, randomly selected community population. We also examined whether vitamin cofactors and the C677T genetic mutation of the methylenetetrahydrofolate reductase (MTHFR) enzyme were major contributors to elevated plasma tHcy and carotid vascular disease.

Methods and Results—In 1111 subjects (558 men, 553 women) 52±13 years old (mean±SD; range, 27 to 77 years) recruited from a random electoral roll survey, we measured fasting tHcy and performed bilateral carotid B-mode ultrasound. For the total population, mean tHcy was 12.1±4.0 μmol/L. Plasma tHcy levels were correlated with IMT (Spearman rank \( r_s = 0.31, P = 0.0001 \)). After adjustment for age, sex, and other conventional risk factors, subjects in the highest versus the lowest quartile of tHcy had an odds ratio of 2.60 (95% CI, 1.51 to 4.45) for increased IMT and 1.76 (95% CI, 1.10 to 2.82) for plaque. Serum and dietary folate levels and the C677T mutation in MTHFR were independent determinants of tHcy (all \( P = 0.0001 \)). The mutant homozygotes (10% of the population) had higher mean tHcy than heterozygotes or those without the mutation (14.2 versus 12.3 versus 11.6 μmol/L, respectively, \( P = 0.0001 \)). The inverse association of folate levels with tHcy was steeper in the mutant homozygotes. Despite this, the C677T MTHFR mutation was not independently predictive of increased carotid IMT or plaque formation.

Conclusions—Mild hyperhomocysteinemia is an independent risk factor for increased carotid artery wall thickness and plaque formation in a general population. Lower levels of dietary folate intake and the C677T mutation in MTHFR are important causes of mild hyperhomocysteinemia and may therefore contribute to vascular disease in the community.

Key Words: homocysteine ■ genes ■ folate ■ atherosclerosis ■ ultrasonics

A number of studies, recently reviewed by Boushey et al, have linked elevated plasma total homocysteine (tHcy) levels to atherosclerotic vascular disease affecting coronary, carotid, and peripheral arteries. Several mechanisms for homocysteine-associated vascular disease have been proposed, including potentiation of atherosclerosis through endothelial dysfunction, smooth muscle cell hyperplasia, and increased formation of oxidized lipids. Previous carotid B-mode ultrasound studies have suggested that hyperhomocysteinemia is a risk factor for carotid atherosclerosis, but these data are limited to a single case-control study and cross-sectional studies in older populations.

 Plasma tHcy levels are affected by dietary intake of vitamin cofactors in homocysteine metabolism, especially folate, vitamins B, and B. Genetic mutations of key metabolic enzymes, creatine-creatinine synthesis, and renal elimination. A genetic mutation (nucleotide C677T, alanine [α] to valine [v] substitution) in the enzyme methylenetetrahydrofolate reductase (MTHFR), which renders the enzyme thermolabile and functionally impaired, has recently been described. Because this enzyme is involved in the folate-dependent remethylation of homocysteine to methionine, a
reduced efficiency may be an important determinant of elevated tHcy in the general population.

To further evaluate the contribution of elevated tHcy to atherosclerotic vascular disease, we examined the independent association between fasting tHcy and carotid artery intima-media thickening and focal plaque formation in a large, randomly selected community population, with a broad age range (27 to 77 years) and an equal male-to-female ratio. We also examined whether the C677T MTHFR mutation and vitamin cofactors were important determinants of plasma tHcy and carotid vascular disease in this population.

Methods

Subjects

Subjects were original participants in the 1989 Australian National Heart Foundation (NHF) Perth Risk Factor Prevalence Survey. This was a random electoral roll survey of 2000 people from the Perth, Western Australia, metropolitan area, with equal numbers of men and women and equal numbers of subjects in each decile of age between 20 and 70 years. Repeat electoral roll and death record matching in May 1995 established a current address for 1807 living subjects. All of these were invited to attend our study clinic between June 1995 and December 1996, and 1111 subjects (61% of those eligible) agreed to participate. Subjects who had previous carotid artery surgery were excluded. The age-adjusted prevalence of risk factors in the present study population was similar to that reported for the entire 1989 cohort. Written informed consent was obtained from all study participants. The study protocol was approved by the Institutional Ethics Committee of the University of Western Australia.

Laboratory Measurements

In all subjects, a fasting venous blood sample was obtained. Special care was taken with the samples for tHcy analysis, with the plasma separated by centrifugation shortly after venipuncture and transport in ice-cooled containers. Fasting tHcy was determined by reverse-phase high-performance liquid chromatography after treatment with tributylphosphine, deproteinization, and fluorogenic derivatization by the method of Araki and Sako. The interassay coefficient of variation was 6% in our laboratory. Total cholesterol, HDL cholesterol, and triglyceride levels were determined enzymatically with a Hitachi 747 autoanalyzer. LDL cholesterol was calculated with Friedewald’s method. Plasma creatinine levels were also measured. DNA was extracted by the salt phenol chloroform method from the cells of the buffy coat. MTHFR genotype was determined by HinfI digestion of the PCR products. Serum folate was determined by immunoassay with ACS:180 (Chiron Diagnostics).

Risk Factor and Dietary Assessment

A self-administered questionnaire similar to that used by the 1989 Australian NHF Risk Factor Prevalence Survey was used to record a history of hypertension, hyperlipidemia, diabetes, angina pectoris, myocardial infarction, stroke, or a family history of premature-onset coronary or cerebrovascular disease (by age 55 years) in first-degree relatives. Smoking lifetime exposure by pack-years was calculated. Anthropomorphic measurements and the lower of 2 resting sitting blood pressures, measured with a mercury column manometer, were recorded by a trained research nurse. All subjects completed a self-administered semiquantitative food-frequency questionnaire, recorded by a trained research nurse. All subjects completed a self-administered semiquantitative food-frequency questionnaire prepared by the CSIRO Division of Human Nutrition, Australia, with responses reviewed by a research nurse on the day of their visit. The average daily intake of folate and vitamins B6 and B12, adjusted for supplemental vitamin use, was calculated by the CSIRO Dietary Assessment by Computer Program.

Figure 1. B-mode ultrasound of distal common carotid artery demonstrating measurement of IMT as distance from lumen-intima (leading edge 1) to media-adventitia (leading edge 2) interfaces of far wall measured over a 1-cm segment length. Mean IMT is average of 6 measurements from right and left common carotid arteries. A, Normal IMT; B, increased IMT.

Carotid Ultrasound Examination

Bilateral carotid B-mode ultrasound was performed by 2 trained sonographers using a 7.5-MHz annular phased-array transducer on an Interspec (Apogee) CX 200 ultrasound machine. Scans were performed according to a standardized protocol similar to that used by Salonen et al. The characteristic echo interfaces on the far wall of the distal common carotid artery were optimized and recorded on super VHS videotape, along with an ECG lead, for subsequent offline analysis. A thorough search of the distal common carotid artery, carotid bulb, and internal and external carotid arteries was also made to determine the presence of focal plaque and/or calcific deposits. Plaque was defined as a clearly identified area of focal increased thickness (>1 mm) of the intima-media layer.

The intima-media thickness (IMT) was defined as the distance between the characteristic echoes from the lumen-intima and media-adventitia interfaces, as shown in Figure 1. End-diastolic images were digitized, and a semiautomated edge-detection software program was used to identify leading-edge echo-interface points from the far wall of the distal 1 cm of the common carotid artery. Three end-diastolic images were analyzed from both the right and left distal common carotid arteries at a site free of any discrete plaque, and measurements were averaged to give the mean IMT. Repeat measurement of randomly selected scans revealed no significant variation in the IMT measurement obtained during any specific time period of the study. Quality control measures included repeat scans on a subset of 30 subjects on 2 separate occasions 7 to 10 days apart. The intraobserver coefficient of variability was 2.9% for sonographer 1 and 4.8% for sonographer 2. The interobserver coefficient of variability was 5.9%.

Statistical Analysis

Mean IMT was treated as a continuous as well as a categorical variable, with those above the 80th percentile of IMT for the total cohort (>0.8 mm) classified as increased IMT. Spearman rank correlations were used to describe the association of continuous risk factors, including tHcy, with mean IMT. Determinants of tHcy were assessed by stepwise linear regression. Logarithmic transformation of some biochemical variables, including tHcy, was performed to normalize the distribution. Stepwise logistic regression was used to test the independent relation between sex-specific tHcy quartiles (independent variable) and increased IMT or focal plaque (dependent variables). The tHcy quartiles were entered into the regression analysis, and odds ratios were estimated after adjustment for other confounding risk variables. Analysis was performed with SAS statistical software. ANOVA was used to compare mean values between groups, and if overall significance was demonstrated,
Results

Subjects

The clinical characteristics of the study population divided into men (n=558) and women (n=553) are shown in Table 1. A similar prevalence of conventional risk factors was observed between men and women, except that men had higher blood pressure and waist-to-hip ratio, lower HDL cholesterol, and higher triglyceride levels than women (Table 1). A previous history of myocardial infarction or stroke was recorded in 7% of the total population.

**tHcy and Carotid Atherosclerosis**

Figure 2 shows the frequency distribution of plasma tHcy by sex. The mean tHcy concentrations were 12.9±3.1 μmol/L in men (range, 7.6 to 40.5) versus 11.2±3.8 μmol/L in women (range, 6.0 to 35.9) (P=0.001). In men, the cutoff thresholds for tHcy quartiles were ≤10.5, 10.6 to 12.4, 12.5 to 14.4, and >14.4 μmol/L, and in women, they were ≤8.4, 8.5 to 10.5, 10.6 to 12.8, and >12.8 μmol/L.

In the overall population, the average carotid artery mean IMT was 0.71±0.14 mm, and focal plaque was identified in 26% of subjects. Although men had a higher mean IMT than women (0.73±0.14 versus 0.69±0.13 mm, P=0.001), women still composed 41% of subjects with increased IMT and 43% with focal plaque. Plasma tHcy correlated to IMT (Spearman rank r=0.31, P=0.0001), with a strength of association similar to that of most other conventional risk factors (Table 2).

Figure 3 shows that the prevalence of thickened IMT and focal plaque increased progressively across sex-specific tHcy quartiles (P=0.001, test for trend in both men and women). Before tHcy was entered into the model, logistic regression had selected (in order) age, systolic blood pressure, smoking pack-years, LDL cholesterol, waist-to-hip ratio, and hypertension history as independent determinants of increased IMT (all P<0.05). Sex and plasma creatinine were no longer significant predictors once other risk variables were included, although we continued to include sex in the logistic model. For plaque, similar risk variables were selected, with the addition of a history of vascular disease and diabetes mellitus (all P<0.05). After adjustment for all these risk variables, tHcy remained a significant predictor of increased IMT (P=0.0005) and plaque (P=0.018). Table 3 shows the adjusted odds ratios for increased IMT and plaque between sex-specific tHcy quartiles. When the highest and lowest quartiles of tHcy were compared, an increased odds ratio of 2.60 (95%
Hyperhomocysteinemia and Carotid Wall Thickening

Figure 3. Prevalence of increased IMT (A) and focal plaque (B) in men (■) and women (○) across sex-specific plasma tHcy quartiles. Bars indicate 95% CIs. Cut-off thresholds for tHcy in men and women.

TABLE 3. Adjusted Odds Ratios for Increased Carotid Intima-Media Thickness and Focal Plaque According to Sex-Specific Quartiles of Plasma tHcy

<table>
<thead>
<tr>
<th>tHcy Quartile†</th>
<th>OR Adjusted for Age and Sex</th>
<th>OR Adjusted for All Risk Factors*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased intima-media* thickness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>1.37</td>
<td>1.23</td>
</tr>
<tr>
<td>3</td>
<td>1.94</td>
<td>1.68</td>
</tr>
<tr>
<td>4</td>
<td>3.05</td>
<td>2.50</td>
</tr>
<tr>
<td>Focal carotid plaque</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>1.22</td>
<td>1.05</td>
</tr>
<tr>
<td>3</td>
<td>1.46</td>
<td>1.29</td>
</tr>
<tr>
<td>4</td>
<td>2.27</td>
<td>1.76</td>
</tr>
</tbody>
</table>

ORs and 95% CIs are for the respective quartile vs quartile 1. *Risk factors adjusted for are age, sex, systolic blood pressure, LDL cholesterol, smoking, waist-to-hip ratio, hypertension history, vascular disease history, and diabetes. †The cutoff thresholds for sex-specific tHcy quartiles (1 to 4) are ≤10.5, 10.6 to 12.4, 12.5 to 14.4, and >14.4 μmol/L in men and ≤8.4, 8.5 to 10.5, 10.6 to 12.8, and >12.8 μmol/L in women.

TABLE 4. Plasma tHcy Levels According to Quartile of Vitamin Cofactors

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Quartile</th>
<th>tHcy, μmol/L</th>
<th>ANOVA P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate, μg/L</td>
<td>≤5.1</td>
<td>14.0 ± 4.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>&gt;5.1 to 6.5</td>
<td>2</td>
<td>12.1 ± 3.5</td>
<td>*</td>
</tr>
<tr>
<td>&gt;6.5 to 8.2</td>
<td>3</td>
<td>11.6 ± 3.6</td>
<td>*</td>
</tr>
<tr>
<td>&gt;8.2</td>
<td>4</td>
<td>10.4 ± 3.1</td>
<td>*</td>
</tr>
<tr>
<td>Dietary folate, μg/d</td>
<td>≤202</td>
<td>13.0 ± 4.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>&gt;202 to 259</td>
<td>2</td>
<td>12.5 ± 4.2</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;259 to 347</td>
<td>3</td>
<td>11.8 ± 3.5</td>
<td>*</td>
</tr>
<tr>
<td>&gt;347</td>
<td>4</td>
<td>10.9 ± 2.9</td>
<td>*</td>
</tr>
<tr>
<td>Dietary vitamin B₆, μg/d</td>
<td>≤1.5</td>
<td>12.6 ± 4.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>&gt;1.5 to 1.9</td>
<td>2</td>
<td>12.3 ± 3.8</td>
<td>NS</td>
</tr>
<tr>
<td>≥1.9 to 2.7</td>
<td>3</td>
<td>12.2 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;2.7</td>
<td>4</td>
<td>11.2 ± 3.8</td>
<td>*</td>
</tr>
</tbody>
</table>

*Multiple-range testing indicates a significant difference between this quartile and quartile 1. tHcy values are ±SD.

There was a strong graded inverse association between tHcy and quartiles of serum folate and dietary folate (ANOVA P = 0.0001), as shown in Table 4. A similar inverse association was found between tHcy and dietary intake of vitamin B₆ (ANOVA P = 0.0001) and vitamin B₁₂ (ANOVA P = 0.026), although the difference was primarily between the lowest and highest quartiles of vitamin intake.

The C677T mutation of MTHFR was present in heterozygous (vv) form in 47% (n = 519) and homozygous (vv) form in 10% (n = 111) of subjects. Genotype frequencies conformed to the Hardy-Weinberg equilibrium. There was no difference in the prevalence of the C677T mutation in men compared with women or between quartiles of vitamin intake. Mutant homoyzgotes had a higher mean tHcy level than heterozygotes or those without the mutation (14.2 versus 12.3 versus 11.6 μmol/L, respectively, P = 0.0001). The mean serum folate level was also lower in vv than in aa homoyzgotes (6.3 versus 7.8 μg/L, respectively, P = 0.0001).

Figure 4 shows that for each genotype (aa, av, vv) there was an inverse relationship between tHcy level and folate quartile (all P < 0.025 for trend), but this was steeper for the vv homoyzgotes. The mean difference in tHcy level from the highest to the lowest quartile of serum folate in vv homoyzgotes was 5.1 μmol/L (95% CI, 1.6 to 8.6 μmol/L) compared with 2.1 μmol/L (95% CI, 1.2 to 3.0 μmol/L) in aa subjects. A similar interaction between MTHFR genotype and dietary folate intake was observed. However, subjects with serum folate levels in the highest quartile displayed no significant differences in tHcy levels between MTHFR genotypes.

Independent determinants of tHcy concentration selected by stepwise linear regression were (in order) serum folate, age, plasma creatinine, dietary folate intake, MTHFR geno-
Figure 4. Plasma tHcy levels according to MTHFR genotype across quartiles of serum folate concentration (A) and dietary folate intake (B). Bars indicate 95% CIs. Difference in mean tHcy concentration from highest to lowest folate quartile is steeper in aa (n=111) than in av (n=519) and vv genotypes (n=481). Cutoff thresholds for serum folate quartiles (1 to 4) were ≥5.1, 5.2 to 6.5, 6.6 to 8.2, and >8.2 μmol/L. Corresponding dietary folate quartiles (1 to 4) were ≥202, 203 to 259, 260 to 347, and >347 μg/d.

tHcy and Carotid IMT

There is good biological evidence to suggest that elevations of tHcy may promote the development of atherosclerosis.1,2 We used B-mode carotid ultrasound as a well-validated technique for the detection of subclinical atherosclerosis.20–22 In particular, carotid wall thickening measured by B-mode ultrasound has been shown to correlate strongly with standard risk factors and focal plaque formation.20–22 Previously, the Atherosclerosis Risk in Communities study,4 from a case-control sampling of study participants 46 to 64 years old, reported an increased odds ratio for thickened IMT for subjects in the top compared with the bottom quintile of tHcy. However, this association was no longer significant after adjustment for conventional risk factors. Sellhub et al.5 in a cross-sectional study of 1041 elderly subjects 67 to 96 years old, demonstrated a 2-fold increase in the risk of carotid stenosis (≥25%) for subjects in the highest versus lowest quartile of tHcy after adjustment for conventional risk factors. Most recently, the Rotterdam Study,6 in a cross-sectional study of 630 subjects ≥55 years old, demonstrated an increased prevalence of thickened IMT, but not plaque, in subjects whose tHcy was in the upper 15th percentile.

We have extended these observations by studying a large, randomly selected general population, equally distributed between men and women and across a broad age range. We found that tHcy levels correlated with mean IMT with a strength of association similar to that of most traditional risk factors. More importantly, we found that there was an independent association of tHcy with increased IMT and plaque, even after adjustment for age, sex, and other conventional risk factors. The cutoff values for the upper tHcy quartiles in our population are in line with those indicated to be associated with an increased risk of vascular disease.1,5 Although we studied a younger population, the odds ratios for increased IMT (2.60) and focal plaque (1.76) for subjects in the highest versus lowest tHcy quartile were similar to those found for carotid stenosis in the elderly Framingham Heart Study cohort.5

tHcy, Vitamin Cofactors, and MTHFR Mutation

We found a strong graded inverse association between tHcy concentration and the level of serum folate and dietary folate intake, similar to that noted by others.7 From the lowest to highest quartile of serum and dietary folate level, there was an average of 2 to 4 μmol/L change in mean tHcy concentration, in accordance with the difference suggested by Boushey et al.1 There was also an inverse but weaker association of vitamin B6 and B12 intakes with plasma tHcy level. The 10% prevalence of homozygotes for the MTHFR C677T mutation was in accord with the 10% to 14% reported in other populations.10,23,24 Mutant vv homozygotes had significantly higher tHcy levels than av heterozygotes or aa homozygotes. Because MTHFR is involved in the folate-dependent remethylation of homocysteine, a low serum or dietary folate level was found to exacerbate hyperhomocysteinemia associated with this mutation. Conversely, increased folate intake could counter the effect of this mutation, because there was no difference in tHcy levels between genotypes in those who had a high serum folate concentration. A degree of folate “wastage” was also suggested by a lower mean serum folate level in the vv subjects relative to the other genotypes despite equivalent dietary folate intakes, a finding also reported by Harmon et al.23 Thus, individuals with the homozygous mutant MTHFR allele may require a higher than usual folate intake to correct the associated hyperhomocysteinemia.

Some case-control studies have reported that homozgyosity for the C677T mutation was associated with premature coronary artery disease,25,26 whereas other studies have not found an association.24,27,28 Although the MTHFR genotype and serum folate were significant determinants of tHcy, together accounting for ≈15% of the total variance in tHcy,
levels, they were still not independent predictors of increased carotid IMT or plaque in our study population. This is not surprising, given that the link between vascular disease and the C677T mutation or folate is likely to be mediated in large part through their effects on homocysteine metabolism. This is also the case for creatinine, which, despite being a significant determinant of tHcy level, was not an independent predictor of increased IMT. Furthermore, the MTHFR mutation will most likely have greater impact on vascular disease in populations that are more folate-deficient.

In conclusion, our study adds further weight to the evidence that tHcy is an independent graded risk factor for atherosclerosis in a general population. Thus, measurement of plasma tHcy may contribute to vascular risk assessment in individuals as well as in population studies. Lower dietary intake of folate and the C677T MTHFR mutation are important causes of mild hyperhomocysteinemia in the community. Although the impact of proposed folate supplementation programs remains to be studied, it is likely that adoption of measures to increase dietary folate intake sufficiently to lower plasma tHcy levels in the general community will have favorable public health effects. The C677T MTHFR mutant genotype may need to be taken into account in decisions on optimal doses of folic acid required to lower the tHcy concentration.

Acknowledgments

This study was supported by grants-in-aid from the National Heart Foundation of Australia (G 94P 4232) and Healthway, the Western Australian Health Promotion Foundation. We appreciate the technical assistance provided by Elsie Yu, Katherine Loh, and Marcus Sommerville, Heart Research Institute, Sir Charles Gairdner Hospital and Clive Hunt, Christine Chin, and Jody Burley, PathCentre, Queen Elizabeth II Medical Center.

References


15. Baghurst KL, Record SJ. A computerised dietary analysis system for use with diet diaries or food frequency questionnaires. Community Health Studies. 1984;8:11–18.


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_Circulation_. 1999;99:2383-2388
doi: 10.1161/01.CIR.99.18.2383

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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